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1. Your reference

P92787GB

20 JAN 2000

2. Patent application number

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0001172.6

NEWPORT

3. Full name, address and postcode of the or of each applicant (underline all surnames)

Res-Del International Limited
33 Market Place
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Oxon RG9 2AA

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

WAKES & 781 974 001
ENGLAND A/L 2.2.00

4. Title of the invention

PHYSIOLOGICAL MEDIUM FOR PERFUSING,
PRESERVING AND STORING ISOLATED CELL,
TISSUE AND ORGAN SAMPLES

5. Name of your agent (if you have one)

Urquhart-Dykes & Lord (Reading)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

1 Richfield Place
Richfield Avenue
Reading RG1 8EQ

Patents ADP number (if you know it)

1644027

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Country

Priority application number
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Number of earlier application

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Yes

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Priority documents	
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Statement of inventorship and right to grant of a patent (Patents Form 7/77)	
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Request for substantive examination (Patents Form 10/77)	
Any other documents (please specify)	

11. I/We request the grant of a patent on the basis of this application.

Signature	<i>Urquhart, Dykes & Lord</i>	Date	17 January 2000
URQUHART-DYKES & LORD			

12. Name and daytime telephone number of person to contact in the United Kingdom

PAUL WOLFF 0118 950 9937

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PHYSIOLOGICAL MEDIUM FOR PERFUSING, PRESERVING AND STORING ISOLATED CELL, TISSUE AND ORGAN SAMPLES

The present invention relates to the formulation of a physiological liquid medium having the basic, synergistic components to allow its universal application in preserving cellular and functional integrity *in vitro* of different cell, tissue and organ types isolated from different mammalian species.

Historically, the design of physiological perfusion solutions dates back to the thesis proposed by the French physiologist, Claude Bernard in the eighteenth
seventies who put forward his theory on the *milieu intérieur*, basically purporting that to maintain the *whole* (person) one should ensure that the surrounding extracellular environment should be balanced in all respects. Unfortunately, the misinterpretation or misconception of Bernard's *milieu intérieur* has led researchers to confuse the *extracellular* with the *intracellular* phases of cell function and largely to overlook the need to maintain the cell as a *whole* entity. The basic *salt* solutions currently used for *in vitro* or *isolated* organ/tissue studies derive from the simple formulation used by Sidney Ringer for the isolated perfused frog heart. Similar, empirically contrived basic salt solutions have been utilised for isolated mammalian preparations. The conventional use of phosphate/bicarbonate buffered salines was instigated by Krebs et al for studies on isolated *homogenates* of mitochondria, ie. *intracellular* organelles, from pigeon liver. Later, Krebs, in his classical paper on the analysis of oxygen consumption in tissue slices from different organs in a variety of animal species, acknowledged that substrate depletion in isolated tissue/organ preparations *over time* was a consideration that had not

been addressed in the composition of previous physiological solutions. A correct interpretation of Bernard's hypothesis necessitated that the *whole* cell should subtend metabolic homeostasis.

5 Traditionally, phosphate/bicarbonate buffers have been used for sixty years and are still currently used, with questionable validity, in perfusion/preservation solutions for mammalian and human tissues/organs. It is of interest to note that it has been known for 40 years that *inorganic* phosphate ions inhibit glycolysis and oxidative phosphorylation, creatine
10 kinase and the enzymes involved in oxygen free radical scavenging, the latter being implicated in reperfusion injury and oedema formation in numerous organ systems. Maintenance of pH over time is further complicated by the instability of phosphate-buffered perfusion and preservation solutions caused by the precipitation of calcium phosphate and bicarbonate, accentuated by the
15 change in their dissociation constants over the temperature range 4 - 37°C.

The present invention provides an answer to the sill-existing need to provide a solution to overcome the deleterious effects of inorganic phosphate ions on cell metabolism and associated cellular function while also serving to augment
20 the natural physiological processes essential to preservation of isolated cell, tissue and organs from mammalian species, for instance during the transport of organs for transplant. Such organs deteriorate rapidly, and many useful organs cannot be used because too much time elapses between collection and delivery to an intended recipient. The solution according to the present
25 invention extends the safe period.

The present invention provides a physiological medium which comprises an aqueous solution in sterile purified water of:

- 30 (i) a salt component comprising:

- (a) from 100 to 150 mmoles /L of sodium ions,
- (b) from 2.5 to 6.2 mmoles /L of potassium ions,
- (c) from 1.0 to 2.5 mmoles /L of calcium ions,
- (d) from 0.4 to 2.4 mmoles /L of magnesium ions, and
- (e) from 96 to 126 mmoles /L of chloride ions;

(ii) a buffer component comprising

- (f) from 21 to 27 mmoles /L of bicarbonate ions, and
- (g) from 1 to 12 mmoles /L of TES, MOPS or BES;

(iii) a substrate component comprising:

- (h) 2 to 11 mmoles /L of glucose
- (i) 50 to 150 μ moles /L of glycerol and
- (j) 7 to 15 μ moles /L of choline;

(iv) an amino acid component comprising:

- (k) 5 to 400 μ moles /L of glutamate
- (l) 5 to 200 μ moles /L of aspartate and
- (m) 100 to 2000 μ moles /L of glutamine;

(v) a co-enzyme component comprising:

- (n) 1 to 120 nmoles /L of thiamine cocarboxylase;

(vi) a vitaminoid component comprising:

- (o) 40 to 70 μ moles /L of D- or DL- or L- carnitine;

(vii) a protein component comprising:

(p) 5 to 200 m I.U./L of porcine or human insulin; and

5 The present invention also provides a method for producing a physiological medium as described above which comprises adding in the following order: sodium chloride, potassium chloride, calcium chloride, magnesium chloride, the TES, MOPS, or BES, thiamine, carnitine, choline, glycerol, insulin, aspartate, glucose, glutamate, glutamine, and sodium bicarbonate to sterile purified
10 water, with constant stirring, making up to the desired volume, filtering and storing in sterile sealed vessels.

It will be noted that the compositions according to the invention contain no animal derived serum protein, such as foetal bovine serum or bovine serum
15 albumin, which have been banned by the FDA for such applications in single cell or human tissue/organ biotechnology.

Buffer components:

With regard to the components of the solutions according to the invention
20 a natural physiological buffer system, namely NaHCO_3 / pCO_2 , has been adopted for the solution in accordance with the invention, in combination with the zwitterionic Good's buffer, BES, which acts by virtue of its ideal pK_a over a temperature range of 10-37°C, to provide a stable pH, an essential requisite for cellular preservation. BES has been shown to be non-toxic to even
25 cultured mammalian cells in long-term studies and exhibits negligible binding of Ca^{2+} or Mg^{2+} , so removing the potential hazard of precipitation of divalent ions which occurs when using conventional bicarbonate/phosphate or double phosphate buffer solutions. Indeed, 10x concentrates of solutions according to the invention have been experimentally shown to have a shelf-life (stored
30 at 3-8°C) in excess of 14 months. As alternatives to the use of N,N-bis-(2-

hydroxyethyl)-2-amino ethanesulfonic acid (BES), it is possible to use morpholinopropane sulfonic acid (MOPS) or N-tris-hydroxymethyl methyl-2-amino ethanesulfonic acid (TES).

5 A major distinguishing feature of solution in accordance with the invention in comparison to conventional perfusion salines is the absence of inorganic phosphate which, for the past 80 years, has been used as the buffer vehicle in conventional perfusion solutions, even though inorganic phosphate radicals have been shown over the last 40 years to inhibit glycolysis and oxidative phosphorylation, creatine kinase activity, and free radical scavenging enzymes.

10 Inorganic phosphate has been reported to have an inhibitory effect on glycolysis by interfering with the co-operativity of Mg^{2+} with the rate limiting enzymes, hexokinase and phosphofructokinase, and on the solubility of
15 creatine kinase. Numerous investigators, in an attempt to compensate for the inhibitory effect upon glycolysis and associated decline in physiological performance produced by phosphate buffered salines, have included pyruvate at non-physiological serum levels (i.e. 2.0 mmoles vs 0.2 mmoles) in their perfusate salines. Experiments conducted on the isolated, perfused/perifused
20 rat liver have shown that leakage of lactate dehydrogenase (LDH) occurred after only 10 minutes with Krebs & Henseleit saline but not for 300 minutes with a solution in accordance with the invention. Under the latter conditions, the further addition of pyruvate would be contraindicated as pyruvate is known to cause inhibition of the H_4 and H_3M subunits of LDH in the liver and,
25 incidentally, the heart. Again, it may be emphasised that for the preservation of isolated tissues/organs, all attempts should be made to retain metabolic homeostasis at the cellular level by maintaining 'autoregulation' of enzymatic function.

30 The preferred salt component comprises:

135.32 mmoles /L of sodium ions, 5.00 mmoles /L of potassium ions, 1.25 mmoles /L of calcium ions, 0.45 mmoles /L of magnesium ions, as chloride salts, and 118.40 mmoles /L of chloride ions as sodium, potassium, calcium and magnesium salts.

5

The preferred buffer component comprises:

25.00 mmoles /L of bicarbonate ions as sodium salt and 5.0 mmoles /L of N,N-bis (2-hydroxy ethyl)-2- amino-ethanesulfonic acid (BES).

10

Substrate components

The solution according to the invention provides a number of essential substrates to retain metabolic homeostasis of the isolated organs/tissues. Glucose and glycerol have been shown to be satisfactory in meeting the energy demands of isolated tissues and organs, even when they are not the preferred substrate for the organ (e.g. the heart) by the inclusion of *physiological* levels of insulin. Apart from their ability to be metabolised, glycerol and glucose also have free radical scavenging and membrane stabilizing properties, which have been shown to be extremely important in maintaining the physiological viability of isolated tissues and organs. Preferably, the substrate component comprises 10 mmoles /L of D-glucose, 110 μ moles /L of glycerol and 10.0 μ moles /L of choline as the chloride salt.

20

Amino acid components:

Aspartate and glutamate have also been included in solutions according to the invention to enhance oxidative metabolism by replenishing TCA cycle intermediates, thereby maintaining high energy phosphate levels even during ischemic insult. Similarly, glutamate is involved in maintaining intracellular oxidation-reduction potentials. Essentially, it is suggested that, by optimising

25

the aspartate-malate and glycerol phosphate shuttles, cells will maintain an optimal NAD/NADH balance and thereby sustain adenine nucleotide levels.

Preferably, the amino acid component comprises 300 μ moles /L of L-glutamate as sodium salt, 20 μ moles /L of L-aspartate as sodium salt and 400 μ moles /L of L-glutamine.

Co-enzyme components:

Thiamine cocarboxylase plays an essential role in the oxidation of α -keto acids, and is included in the compositions to prevent the accumulation of pyruvate and pyruvate aldehyde and thereby cell toxicity.

In the tricarboxylic acid cycle, TPP is a co-factor in the metabolism of α -ketoglutaric acid to form succinyl-coenzyme A, by oxidative decarboxylation, or to form glutamate, by reductive amination.

In essence, TPP is involved in numerous interrelated, biochemical pathways, especially those of the Pentose Phosphate and Glycolytic pathways.

The thiamine may, for instance, be employed as thiamine pyrophosphate or as thiamine diamide.

Preferably the coenzyme component comprises 40.0 nmoles /L of thiamine as thiamine pyrophosphate chloride.

Vitaminoid component

The vitaminoid, carnitine, has been reported to have multiple effects in improving cardiac function other than by simply optimising oxidative metabolism, such as, by promoting the utilization of alternative substrates and may additionally improve coronary blood flow. L-carnitine is preferred to the

D- or DL-isomers, because it causes no inhibition of acetyl co-enzyme A / free fatty acid metabolism. Preferably the vitaminoid component comprises 50.0 μ moles /L of $[-]\beta$ -hydroxy- γ -trimethylamino-butyrate hydrochloride (L-carnitine). In this invention the inclusion of the L-isomer of carnitine in the formulation of the solution was intended to optimise the transport of long chain fatty acids from the cytosol into the mitochondrial matrix to the site of β -oxidation and thereby to buffer the intramitochondrial acetyl CoA/CoA ratio by stimulating the synthesis of acetyl carnitine from carnitine acetyltransferase. This reduction in the ratio of acetyl CoA/CoA will result in an efflux of acetyl carnitine from the mitochondria with an associated stimulation of pyruvate dehydrogenase and reversal of fatty acid inhibition of glucose oxidation. Ultimately, the optimisation of free fatty acid utilisation as an energy source is essential for all types of cells but this must be done with preservation of carbohydrate (glucose) utilisation by optimised functioning of the enzymes involved in glycolysis, eg. hexokinase, glucokinase, phosphofructokinase.

D- or DL- isomers of carnitine are less effective in fulfilling this function.

Insulin components:

The use of human recombinant insulin (expressed in E. Coli) not only precludes the risk of antigenic or viral contamination in recipient cells/tissues/organs, as may be the case with insulin derived from other mammalian or animal species, but leads to a better fit being achieved of insulin molecules to human insulin receptor structure, ie. receptor specificity will be optimised to retain the many associated functions of insulin in cellular processes.

Preferably the protein component comprises 28.0 m. I.U./L of recombinant human insulin (expressed in E.coli).

Few of the disclosed formulations for perfusate and preservation solutions over the last 60 years having included insulin, and those that have, made use of insulin at unnatural levels, e.g. $10-50 \times 10^6$ mIU/L (i.e. about a million times more concentrated than in the compositions according to the invention).

5 The reason for this relates to the fact that only a small amount of the insulin exists as single molecules in such concentrations. The rest of the insulin exists as large aggregates of insulin molecules which are ineffective in action, i.e. individual molecules of insulin are needed to stimulate insulin receptors on cell membranes.

10 Essentially, the biochemical effects of insulin do not simply relate to its ability to regulate carbohydrate metabolism and facilitated transport of circulating glucose into cells but also (i) the enhancement of intracellular glucokinase activity and amino-acid incorporation into proteins, (ii) stimulation of DNA
15 translation, (iii) increased lipid synthesis, and (iv) stimulation of sodium, potassium and inorganic phosphate into cells.

The invention acknowledges that in the absence of species-specific insulin there will be profound changes in the entire balance of cellular metabolism, eg.
20 increased gluconeogenesis from protein, increased lipolysis and ketogenesis. The net result will be a total disruption in metabolic homeostasis and cellular function.

25 The use of human recombinant insulin in the compositions according to the invention, in preference to animal serum-derived insulin, relates not only to meeting FDA regulations but to the fact that a better fit will be achieved of the insulin to the human insulin receptors, i.e. receptor specificity will be optimised.

Therefore, normal serum levels of insulin have been utilised in the compositions according to the invention and this could only be achieved by acidifying the insulin to prevent aggregates of insulin forming so allowing individual molecular species of insulin to exist in solution.

Using alkali or neutral pH solutions do not achieve this molecular dispersion of insulin molecules.

Antibiotic components:

Preferably, an antibiotic component is included to ensure that, should there be any accidental contamination by micro-organisms during transport, any multiplication of such micro-organisms can be prevented. Preferably, the antibiotic component comprises 10 to 150 mg/L, more preferably 100 mg/L, of D-[-]-theo-2-dichloroacetamide-1-(p-nitrophenyl)-1,3-propane acid (chloramphenicol). Other antibiotics may be used, but care must be taken to ensure that the particular antibiotic employed does not interfere with the tissues or organs being stored or transported.

Ionic:

The concentrations of ionic species in solutions according to the invention acknowledges the activity coefficients of each ionic species and not simply their total serum concentrations. For instance, serum binding of Ca^{2+} and Mg^{2+} must be distinguished from the actual free, ionised levels of these ions. Magnesium ions are important in a number of critical cellular reactions and their extracellular presence is reported to stimulate mitochondrial respiratory activity and modulate the effects of rapid calcium influx and potassium efflux. Equally, an adequate concentration of calcium ions must be present in the preservation solution to avoid the calcium paradox, observed upon subsequent exposure of the donor organ to total serum calcium levels upon reperfusion and transplantation. Additionally, the ionic conductivity of solutions according

to the invention is comparable to that of human serum, namely 12.6 mS cm^{-1} and as such maintains the ionised status of the cell membrane and activities of enzymic moieties. Of significant concern in the majority of preservation solutions are the levels of potassium and sodium, in that the potassium ion concentration is some twenty-five times higher and sodium five to fifteen times lower than their serum levels.

Osmolarity:

A solution according to the invention is isosmotic to human serum (ca. 290 mOsmoles/L) and does not appear to necessitate the inclusion of plasma expanders, as demonstrated by the fact that only minor changes (ca. 8%) in hydration occur during long term (i.e. 4-52 h) hypothermic perfusion of the isolated rat heart and visceral nerve-muscle preparations. This may be explained by the fact that the cell membrane lies in continuity with a 99% gel interstitial phase so providing natural colloidal buffering to excess Donnan ionic equilibrium exchange across the cell membrane. The majority of the osmotic pressure is provided by Na^+ and its accompanying anions and only a small component (ca. 0.5%) can be attributed to plasma proteins and thereby has not justified the inclusion of oncotic agents in solutions according to the invention. The practicality of including oncotic agents, as in other commercially available perfusate/preservation solutions, is further compromised by their affinity for Ca^{2+} and Mg^{2+} , necessitating prior dialysis in fresh solution so as not to disturb the cationic composition of the perfusate. The labile nature of polypeptide expanders also makes them impractical through their predisposition to mechanical denaturation as occurs in their preparation and circulation through perfusion apparatus. Unfortunately, while these colloidal expanders are essentially non-toxic, their use in preservation fluids is contraindicated in terms of, for example, (1) raised viscosity increases the thickness of the 'unstirred' layer around cells so hindering diffusion of metabolites, (2) alteration of the surface membrane bioelectric potential so

disrupting cellular metabolism and receptor activities, (3) antigenicity of proteinaceous expanders, (4) agglutination and haemolysis of RBC's and (5) blockage of microvasculature and ischemia.

5 Amongst potential uses for the solutions according to the invention are as a flushing/holding/preserving/transporting medium for Mammalian embryos (among others) for 24-48 hours at 20 to 25°C. In agriculture this could be used for embryos of such species as sheep, goats, deer, cattle, pigs and horses.

10 Another potential use is as a non-frozen solution for use as a medium for storing semen for 24-48 hours at 20 to 25°C. The techniques heretofore used for this purpose are restricted solely to freezing the sperm with highly variable success. The solution is particularly useful for porcine semen since porcine
15 semen cannot be frozen as compared to bovine semen.

In vitro applications in accommodating a variety of animal organ/tissue preparations used in physiological and pharmacological bioassay techniques for scientific experimentation at student to research levels of exploration, e.g.

- 20
- a) perfused (cannulated)/perifused mouse, rat, guinea pig heart/heart-lung, liver, kidney preparations
 - b) perifused visceral muscle preparations, e.g. blood vessels, G.I. tract,
25 reproductive tract biopsies
 - c) perifused mammalian skeletal muscle biopsies, e.g. mouse, rat, rabbit, human
 - d) perifused tissue slices, e.g. liver, brain
30

The preparation of solutions according to the invention is sensitive to the method by which stock solutions are prepared and stored, and to the order in which they are incorporated. The following Example is given as an instance of the preferred method of combining the various ingredients into the compositions.

Example

In the following, thiamine pyrophosphate (cocarboxylase), Sigma C4655 was prepared as a 0.4 mg/mL stock solution in MilliQ (endotoxin-free) purified water, and stored frozen in dark glass vials. Choline chloride (Sigma C7527) was prepared as a 17.5 mg/mL stock solution in MilliQ endotoxin-free purified water and stored frozen in glass vials. Human recombinant insulin (Sigma I0259) was prepared as a 0.5 I.U./mL stock solution in endotoxin-free MilliQ purified water acidified to pH 2.4 with 0.1N hydrochloric acid and stored frozen in glass vials.

In the following preparations, endotoxin-free MilliQ purified water was used throughout, both in the initial stirring, and in the final dilution.

For the preparation, a stainless steel container was filled with 8 litres of MilliQ and, the following ingredients were weighed out and added while constantly stirring, in the following order: 642.96 grams of sodium chloride (CFK0484), 37.28 grams of potassium chloride (BDH10198), 18.38 grams of calcium chloride dihydrate (BDS10117), 9.14 grams of magnesium chloride hexahydrate (BDH101494) and 106.61 grams of BES free acid (Sigma B6266), 1.84 milligrams of thiamine pyrophosphate (Sigma C9655) (using 4.6 mL of the stock solution), 0.9899 grams of L-carnitine (Sigma C0238), 0.1397 grams of choline chloride (Sigma 7527) in the form of 8 ml of the stock solution, 1.013 grams of glycerol (Sigma G2025), 2.8 I.U. of human recombinant insulin (5 ml of the stock solution), 0.310 grams of L-aspartate

sodium salt (Sigma A6683), 180.2 grams of anhydrous D-glucose (Sigma G7021), 5.07 grams of L-glutamate sodium salt (Sigma G5889) and 5.84 grams of L-glutamine (Sigma G5763). The whole was stirred until completely dissolved and then the final volume of 10 litres was produced by adding further MilliQ purified water.

The solution was filtered through a sterile filter (0.2 μ m Sartobran PH) into 100mL sterile sealed glass bottles.

This solution is a 10x concentrate of the solution intended for use. When needed, it can be diluted with the appropriate quantity of MilliQ.

For use as a perfusion and preservation solution, 100ml of the concentrate may be diluted with 900 mL of double deionised or endotoxin-free MilliQ purified water to 1 litre with the addition of 2.1 g of endotoxin-free sodium bicarbonate (Sigma S4019) and stored at 8-10°C prior to use. Sodium bicarbonate is not added to the concentrates before they are stored, since extended storage of the concentrate containing bicarbonate ions may cause precipitations of calcium carbonate.

For use as a perfusion and preservation solution, each litre of solution may contain 100 mg/L of chloramphenicol (Sigma C3175) to prevent the risk of bacterial contamination as may occur during extended periods of *in vitro* experimentation under exposed, atmospheric conditions.

The following factors are very important in the preparation of compositions according to the invention. The most critical factors are:

1. The method of assembly of the solutions according to the invention and, specifically:

2. Use of endotoxin-free MilliQ water to make up all stock solutions and the 10x concentrate bottles of manufactured solutions according to the invention.
- 5 3. The method of preparing sterile stock solutions according to the invention and concentrates not involving autoclaving or irradiation - e.g. irradiation to achieve sterility resulted in degradation of glutamine and probably TPP.
- 10 4. The use of glass bottles for all storage of stock and 10x concentrates of solutions according to the invention.
5. Preparation of solubilised insulin by acidification at pH 2.4 plus storing insulin stock solutions frozen.
- 15 6. Preparation of thiamine pyrophosphate plus TPP stock solutions stored frozen under dark conditions (see reason below).
7. Preparation of choline-chloride plus stock solutions stored frozen.
- 20 8. Use of magnesium chloride hexahydrate (i.e. $6 \text{ H}_2\text{O}$). This is because if the dehydrate salt is used then as it adsorbs water the weight used to calculate the precise magnesium ion content will be in error - this is a common reason for wrongly made up Krebs solutions in terms of
- 25 correct Mg-ion and Ca-ion levels.

It is not possible to omit any of the preferred components when formulating solutions according to the invention. All of these components work in synergy to produce the overall balanced physiological effect. This is why the use of

human insulin and L-carnitine will only add to the overall attainment of viability of this versatile mammalian liquid medium.

Manufacture:

1. Stock solutions: Various stock concentrations of solutions according to the invention namely, 1x, 10x and 20x for long-term storage have been prepared and trialed successfully, but the preferred stock concentrates are 10x concentrates using endotoxin-free milliQ water and sterile filtered into sealed 100mL bottles for storage under dark conditions at 3-8°C. Stock solutions are reconstituted for use as 1x concentrate solutions by the addition of 100mL of 10x concentrates of stock solutions to 900mL of double deionised or endotoxin-free MilliQ purified water with the addition of 2.1g of sodium bicarbonate to give a final pH of 7.22 ± 0.04 at 20°C. Sterile stock 10x concentrations of solutions according to the invention have a pH of 4.6 ± 0.2 and have been shown to be retained as such for periods of up to five years. The recommended manufactured shelf-life of 10x stock concentrates of solutions according to the invention is 14 months when stored at 3-8°C.
2. Cocarboxylase: Stock solutions of thiamine pyrophosphate chloride (cocarboxylase) are prepared at 18.4 g/mL using endotoxin-free milliQ purified sterile filtered into dark sealed vials to prevent the photon degradation of thiamine pyrophosphate and stored frozen prior to the assemblage of 10x stock concentrates of solutions according to the invention.
3. Insulin: Human recombinant insulin is prepared as acidified (pH 2.4) stock concentrated solutions at 0.5 m I.U./mL using endotoxin-free

milliQ purified water and sterile filtered into sealed vials and stored frozen prior to the assemblage of stock concentrates of solutions according to the invention.

- 5 4. Choline: Stock solutions of choline chloride are prepared at 17.45 mg/mL using endotoxin-free milliQ purified water and stored frozen in sealed vials prior to the assemblage of stock concentrates of solutions according to the invention.
- 10 5. Chloramphenicol is not an essential component of solutions according to the invention but is preferably added, either for storage, or after the storage vials have been opened, to ensure sterility during extended exposure of the solutions to the atmosphere, e.g. during perfusion or perfusion, or non-perfused preservation procedures.

CLAIMS

1. A physiological medium which comprises an aqueous solution in sterile purified water of:

- (i) a salt component comprising:
 - (a) from 100 to 150 mmoles /L of sodium ions,
 - (b) from 2.5 to 6.2 mmoles /L of potassium ions,
 - (c) from 1.0 to 2.5 mmoles /L of calcium ions,
 - (d) from 0.4 to 2.4 mmoles /L of magnesium ions, and
 - (e) from 96 to 126 mmoles /L of chloride ions;
- (ii) a buffer component comprising
 - (f) from 21 to 27 mmoles /L of bicarbonate ions, and
 - (g) from 1 to 12 mmoles /L of TES, MOPS or BES;
- (iii) a substrate component comprising:
 - (h) 2 to 11 mmoles /L of glucose
 - (i) 50 to 150 μ moles /L of glycerol and
 - (j) 7 to 15 μ moles /L of choline;
- (iv) an amino acid component comprising:
 - (k) 5 to 400 μ moles /L of glutamate
 - (l) 5 to 200 μ moles /L of aspartate and
 - (m) 100 to 2000 μ moles /L of glutamine;
- (v) a co-enzyme component comprising:
 - (n) 1 to 120 nmoles /L of thiamine cocarboxylase;
- (vi) a vitaminoid component comprising:
 - (o) 40 to 70 μ moles /L of D- or DL- or L- carnitine;
- (vii) a protein component comprising:
 - (p) 5 to 200 m I.U./L of porcine or human insulin; and

2. A physiological medium as claimed in Claim 1 which comprises

(viii) an antibiotic component comprising:

(q) 10 to 150 mg/L of chloramphenicol

3. A physiological medium as claimed in Claim 1 or 2 wherein the salt component comprises:

135.32 mmoles /L of sodium ions, 5.00 mmoles /L of potassium ions, 1.25 mmoles /L of calcium ions, 0.45 mmoles /L of magnesium ions, as chloride salts, and 118.40 mmoles /L of chloride ions as sodium, potassium, calcium and magnesium salts.
4. A physiological medium as claimed in any one of Claims 1 to 3 wherein the buffer component comprises 25.00 mmoles /L of bicarbonate ions as sodium salt and 5.0 mmoles /L of N,N-bis (2-hydroxy ethyl)-2-amino-ethanesulfonic acid (BES).
5. A physiological medium as claimed in any of Claims 1 or 4 wherein the substrate component comprises 10 mmoles /L of D-glucose, 110 μ moles /L of glycerol and 10.0 μ moles /L of choline as the chloride salt.
6. A physiological medium claimed in any of Claims 1 to 5 wherein the amino acid component comprises 300 μ moles /L of L-glutamate as sodium salt, 20 μ moles /L of L-aspartate as sodium salt and 400 μ moles /L of L-glutamine.
7. A physiological medium claimed in any of Claims 1 to 6 wherein the co-enzyme component comprises 40.0 nmoles /L of thiamine as thiamine pyrophosphate chloride.

8. A physiological medium as claimed in any one of Claims 1 to 7 wherein the vitaminoid component comprises 50.0 μ moles /L of [-]- β -hydroxy- γ -trimethylaminobutyrate hydrochloride (L-carnitine).
9. A physiological medium as claimed in any one of Claims 1 to 8 wherein the protein component comprises 28.0 m. I.U./L of recombinant human insulin (expressed in E.coli).
10. A physiological medium as claimed in any one of Claims 1 to 9 wherein the antibiotic component comprises 100 mg/L of D-[-]-theo-2-dichloroacetamide-l-(p-nitrophenyl)-1,3-propane acid (chloramphenicol).
11. A method for producing a physiological medium according to any one of Claims 1 to 10 which comprises adding in the following order: sodium chloride, potassium chloride, calcium chloride, magnesium chloride, the TES, MOPS, or BES, thiamine, carnitine, choline, glycerol, insulin, aspartate, glucose, glutamate, glutamine, and sodium bicarbonate to sterile purified water, with constant stirring, making up to the desired volume, filtering and storing in sterile sealed vessels.
12. Concentrates for the preparation of a physiological medium as claimed in any one of Claims 1 to 10 which comprise the salt, buffer, substrate, amino acid, co-enzyme, vitaminoid and protein components, and dilutable with sterile purified water to form said physiological medium.
13. Concentrates for the preparation of a physiological medium as claimed in any one of Claims 1 to 10 which comprise the salt, buffer, substrate, amino acid, co-enzyme, vitaminoid and protein components, except for sodium bicarbonate, and dilutable with sterile purified water with the addition of sodium bicarbonate to form said physiological medium.

ABSTRACT

PHYSIOLOGICAL MEDIUM FOR PERFUSING, PRESERVING AND STORING ISOLATED CELL, TISSUE AND ORGAN SAMPLES

A physiological liquid medium is provided having the basic, synergistic components to allow its universal application in preserving cellular and functional integrity *in vitro* of different cell, tissue and organ types isolated from different mammalian species.

The medium comprises an aqueous solution in sterile purified water of:

- (i) a salt component comprising:
 - (a) from 100 to 150 mmoles /L of sodium ions,
 - (b) from 2.5 to 6.2 mmoles /L of potassium ions,
 - (c) from 1.0 to 2.5 mmoles /L of calcium ions,
 - (d) from 0.4 to 2.4 mmoles /L of magnesium ions, and
 - (e) from 96 to 126 mmoles /L of chloride ions;
- (ii) a buffer component comprising
 - (f) from 21 to 27 mmoles /L of bicarbonate ions, and
 - (g) from 1 to 12 mmoles /L of TES, MOPS or BES;
- (iii) a substrate component comprising:

- (h) 2 to 11 mmoles /L of glucose
 - (i) 50 to 150 μ moles /L of glycerol and
 - (j) 7 to 15 μ moles /L of choline;
- (iv) an amino acid component comprising:
- (k) 5 to 400 μ moles /L of glutamate
 - (l) 5 to 200 μ moles /L of aspartate and
 - (m) 100 to 2000 μ moles /L of glutamine;
- (v) a co-enzyme component comprising:
- (n) 1 to 120 nmoles /L of thiamine cocarboxylase;
- (vi) a vitaminoid component comprising:
- (o) 40 to 70 μ moles /L of D- or DL- or L- carnitine;
- (vii) a protein component comprising:
- (p) 5 to 200 m I.U./L of porcine or human insulin.